CHAPTER 12

IMPACT OF STRUCTURAL DIFFERENCES ON THE *IN-VITRO* GLUCURONIDATION KINETICS OF POTENTIALLY DOPAMINERGIC HYDROXY-2-AMINOTETRALINS AND NAPHTHOXAZINES USING RAT AND HUMAN LIVER MICROSOMES

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ABSTRACT

The *in-vitro* glucuronidation of seven monohydroxy-2-amino-tetralins and two naphthoxazines has been determined using human-and rat liver microsomes. All these compounds stimulate the dopamine receptor. The influence of the position of the phenolic hydroxyl group was studied with rat microsomes in monohydroxy-2-(N,N-dipropylamino)tetralins. The highest activity and intrinsic clearance was found for 7-OH-DPAT, but the latter values for 5-OH-DPAT and 6-OH-DPAT were much lower by a factor of 9 and 30, respectively. The 8-OH-isomer was not glucuronidated at all.

Substitution of a propyl side chain by a thienylethyl-, or phenylethyl side chain, in 5-hydroxy-DPAT, or in (+)-4-propyl-9-hydroxyhexahydronaphthoxazine (PHNO, N-0500), showed a large increase of the UDPGT affinity and intrinsic clearance especially for N-0437. Also it resulted for N-0437 in a much higher affinity towards the dopaminergic D2 receptor. Although the glucuronidation activity of human microsomes was found to be considerably lower than that of rat microsomes, the latter phenomenon was clearly visible with human microsomes as well.

These findings may have serious implications for the ability of these drugs to adequately reach the brain.

INTRODUCTION

UDP-Glucuronosyltransferase (UDP-glucuronate, ß-glucuronosyl-transferase) is the enzyme conjugating endogenous and exogenous compounds by transfer of glucuronic acid from UDP-glucuronic acid. The enzyme affinity towards xenobiotics is also influenced by the chemical structure of the compound investigated[1-4]. Hydroxy-2-aminotetralins, such as the hydroxy-2-(N,N-dipropylamino)tetralins (DPAT) are known to stimulate dopamine receptors and it has been shown that the position of the phenolic hydroxyl group is - at least partially -responsible for the differences in potency in various dopaminergic test models. The following rank order has been described[5]: 5-OH-DPAT >7-OH-DPAT >6-OH-DPAT > 8-OH-DPAT. Structurally related and even stronger dopamine agonists include 4-propyl-9-hydroxynaphthoxazine (PHNO) and its 4-thienyl-analog (T-PHNO), 5-hydroxy-2-(N-propyl-N-2-phenylethylamino)tetralin (N-0434) and its N-2-thienylamino-analog (N-0437), which are being investigated for use in Parkinson's disease[6-8].

Although these drugs show high affinities towards the D2 receptor, the free phenolic hydroxyl group makes them prone to conjugation reactions, which may seriously affect their bioavailability. Metabolic and kinetic studies with N-0437 in rats have indicated that conjugation with glucuronic acid, forming the ß-linked-0-glucuronide, is by far the major metabolic route. The reaction is *in-vivo* as well as *in-vitro* very fast[9,10].

The metabolic profile in the rat did not indicate formation of a quarternary N-glucuronide. Therefore, we were interested to investigate the impact of structural differences on the *in-vitro* glucuronidation kinetics of a series of hydroxy-2-aminotetralins in order to find an optimum balance between receptor affinity and inactivation by metabolism. In this study we utilized rat liver microsomes and human liver microsomes under virtually identical conditions.

EXPERIMENTAL

Chemicals and reagents

The drugs used in this study were synthesized in our laboratory as described previously[11-14]. Details about the preparation of Cl-N-0437 will be published elsewhere. The compounds were found to be at least 98% pure according to HPLC

and TLC. The drugs which were investigated are:

1. (±)5-hydroxy-2-(N-propyl-N-2-thienylethylamino)tetralin.HCl (N-0437)
2. (±)5-hydroxy-2-(N-propyl-N-2-phenylethylamino)tetralin.HCl (N-0434)
3. trans-(+)4-propyl-9-hydroxynaphthoxazine.HBr (PHNO)
4. trans-(±)4-thienylethyl-9-hydroxynaphthoxazine.HCl (T-PHNO)
5. (±)5-hydroxy-2-(N,N-dipropylamino)tetralin.HCl (5-OH-DPAT)
6. (±)6-hydroxy-2-(N,N-dipropylamino)tetralin.HBr (6-OH-DPAT)
7. (±)7-hydroxy-2-(N,N-dipropylamino)tetralin.HBr (7-OH-DPAT)
8. (±)8-hydroxy-2-(N,N-dipropylamino)tetralin.HBr (8-OH-DPAT)
9. (±)5-hydroxy-8-chloro-2-(N-propyl-N-2-thienylethylamino)tetralin.HCl (Cl-N-0437)

The chemical structures are shown in Fig. 1.

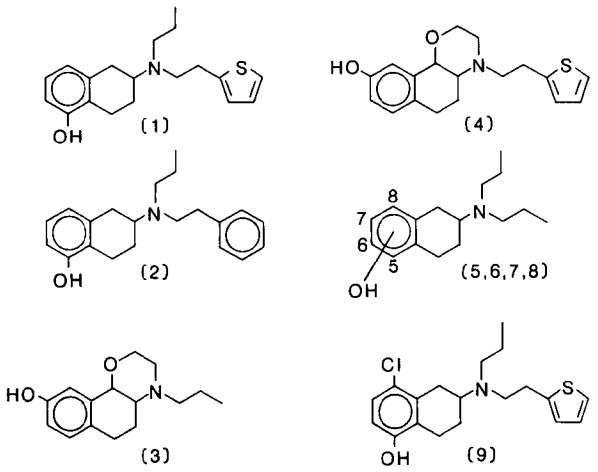

Fig. 1. Chemical structures of the test compounds. See Table I for numbering.

Uridine-5'-diphosphoglucuronic acid (UDPGA) and 1-octanesulfonic acid (sodium salt) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, HPLC grade, was supplied by Baker (Deventer, The Netherlands). All other chemicals used were analytical reagent grade and obtained from Merck (Darmstadt, FRG). Throughout the study de-ionized water was used (Milli-Q purification system, Millipore, Bedford, MA, USA).

Standard solutions

Stock solutions of 2 mM of the different drugs were made in Milli-Q water and were stored at -20°C. After six months no degradation was observed. Appropriate dilutions were made daily in incubation buffer (125 mM Tris HCl, pH 7.4).

Chromatography

The chromatographic system consisted of a model SP 8700 HPLC pump (Spectra Physics, Santa Clara, CA, USA), and a model Rheodyne 7125 injection valve, fitted with a 50 μl sample loop (Rheodyne, Cotati, CA, USA). Detection was performed with a SPD-6A variable wavelength UV detector (Shimadzu Corporation, Kyoto, Japan). Peak heights were recorded with a 3396A reporting integrator (Hewlett Packard, Avondale, PA, USA).

Separations were performed using a stainless steel column 150*4.6 mm i.d., packed with Nucleosil 5 C18 (Macherey-Nagel, Düren, FRG). The isocratic mobile phase contained 10 mM sodium phosphate pH 2.5 and 1-octanesulfonic acid with a final concentration of 2.3 mM. The percentage acetonitrile ranged from 26 to 37%, depending on the drug measured. After filtering the eluent through a 0.20 μm type RC 58 membrane filter (Schleicher and Schuell, Dassel, FRG), the mobile phase was degassed in an ultrasonic bath for 15 minutes. Chromatographic conditions with regard to detection wavelength, percentage acetonitrile and flow rate are presented in Table I.

Table I. Chromatographic conditions used to follow the glucuronidation.

Number	Drug	Detection wave-length (nm)	Acetonitrile in eluent (%)	Flow rate ($ml.min^{-1}$)
1	N-0437	225	32	1.5
2	N-0434	220	35	1.5
3	PHNO	220	26	1.0
4	T-PHNO	225	31	1.2
5	5-OH-DPAT	225	27	1.2
6	6-OH-DPAT	225	27	1.2
7	7-OH-DPAT	225	27	1.2
8	8-OH-DPAT	225	27	1.2
9	Cl-N-0437	229	37	1.5

Microsome preparation

Human liver was kindly donated by the Groningen Liver Cell Research Group. The liver was obtained from a traffic accident victum and stored in small portions at -20°C for several months, until microsome preparation. Male wistar rats (CDL, Groningen, The Netherlands) were killed by cervical dislocation and the livers were rapidly removed. The livers were minced, homogenized at 5°C in a fourfold volume of 0.154 M KCl solution (pH 7.0 by the addition of 0.154 M KOH) by means of a Potter-Elvejehem homogenizer with a teflon pestle (Janke and Kunkel KG, Staufen im Breisgau, FRG). The homogenate was centrifuged at 9,000 g for 20 minutes and the supernatant was centrifuged at 100,000 g for 60 minutes. The microsomal pellet was suspended in 125 mM Tris HCl solution pH 7.4 to give a protein concentration of 7.7 $mg.ml^{-1}$ for the human liver microsomes and 9.2 $mg.ml^{-1}$ for the rat liver microsomes, respectively[15]. These suspensions were stored at -80°C in small vials.

Incubation procedure

Incubations were performed in polyethylene cups (Eppendorf®, 1.5 ml). The medium consisted of 125 mM Tris HCl buffer pH 7.4, 50 mM $MgCl_2$, 2.5 mM UDPGA and 2 $mg.ml^{-1}$ microsomal protein in a final volume of 0.50 ml. Substrate concentrations ranged from 20 to 200 μM for human and 5 to 50 μM for rat liver microsomes.

The enzyme for the glucuronidation, UDPGT, was activated by Mg^{2+} during a

preincubation period of 10 minutes at 37°C in a water bath, with constant shaking[16]. After 5 minutes of preincubation the substrates were added. The reactions were initiated by the addition of the co-substrate UDPGA. The mixtures were incubated at 37°C, usually for 15 minutes, depending on the drug measured, with constant shaking. The reactions were terminated by the addition of 50 μl 0.1 M perchloric acid, and placing the vials on ice. After centrifugation of the incubation mixtures at 1,000 g, 4°C, for 15 minutes a microsomal pellet was obtained. The clear supernatant was decanted and analyzed by HPLC.

Kinetic analysis

The apparent kinetic constants V_{max}, K_m and the intrinsic clearance V_{max}/K_m were determined from a Lineweaver Burk plot.

The Standard Error (SE) was calculated from data obtained from three individual experiments.

RESULTS AND DISCUSSION

Initially, we established that there was a direct quantitative relationship between the formation of the respective glucuronides and the disappearance (0-40%) of the parent compounds. Since it was easier to quantify the parent compounds than the glucuronides, we then chose to follow the glucuronidation reactions indirectly by measuring the disappearance of the parent compounds. Disappearance on the one hand, nor formation of glucuronides on the other, could be observed when UDPGA or microsomal protein were omitted from the incubation medium. Therefore appropriate calibration curves for the different drugs were made in incubation medium without UDPGA. All curves were linear (r^2>0.995) over a concentration range from 5 μM to 200 μM. A typical chromatographic profile before and after the incubation of N-0437 is shown in Fig. 2. The glucuronidation rate, V_{max}, the affinity constant, K_m, and the intrinsic clearance V_{max}/K_m are given in Table II.

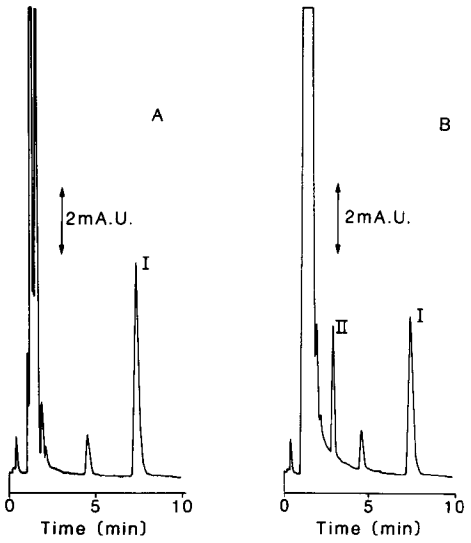

Fig. 2. Typical chromatographic profiles of N-0437 before (A) and after (B) incubation with rat liver microsomes I: N-0437, II: glucuronic conjugate of N-0437.

Fig. 3 shows the Lineweaver Burk plots for those compounds incubated that showed measurable glucuronidation with human liver microsomes, namely: N-0437, N-0434, Cl-N-0437 ant T-PHNO. The four dipropyl-2-aminotetralins and PHNO gave no reaction i.e. the decrease in the parent compound was less than 10% up to three hours of incubation. It should be noted that all substances tested were available as racemates, except for PHNO, which was available as the (+) enantiomer. Stereospecific differences in *in-vitro* glucuronidation has been described for E-10-hydroxy-nortriptyline[17] and for morphine[18]. Only slight differences between the *in-vitro* glucuronidation of (+) and (-) N-0437 were found[19]. Fig. 4 shows the Lineweaver Burk plots for those compounds incubated with rat liver microsomes, that showed measurable glucuronidation, namely N-0437, N-0434, PHNO, T-PHNO, 5-OH-DPAT, 6-OH-DPAT and 7-OH-DPAT. 8-OH-DPAT showed no glucuronidation and Cl-N-0437 was not investigated. In contrast to the results using human liver microsomes, PHNO as well as 5-OH-DPAT, 6-OH-DPAT 7-OH-DPAT were now glucuronidated.

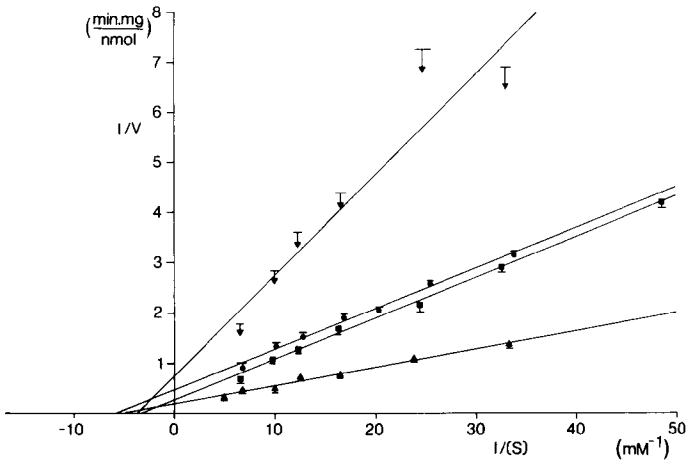

Fig. 3. Lineweaver Burk plots of the test compounds showing glucuronidation using human liver microsomes. Bars indicate standard deviations (■:1, ▼:2, ●: 4, ▲:9).

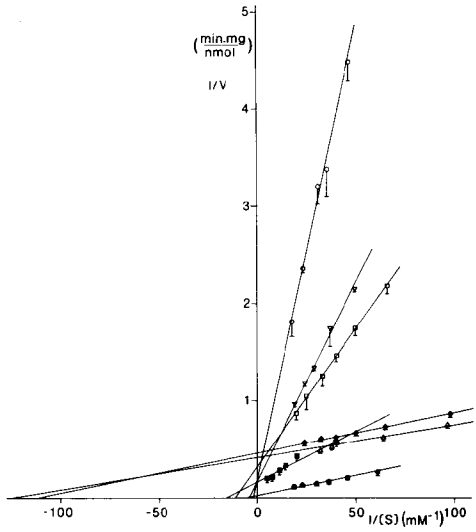

Fig. 4. Lineweaver Burk plot of the test compounds showing glucuronidation using rat liver microsomes. Bars indicate standard deviations (▵:1, ♦:2, ▽:3, ■:4, □:5, ○:6, ●:7).

It is interesting to see that for the various DPAT's both the rate constants and the affinity constants can differ markedly, yet without showing parallelism between the two. Moreover, the intrinsic clearances of the DPAT's show dramatic differences from a very high 311 $\mu l.min^{-1}.mg^{-1}$ for 7-OH-DPAT >5-OH-DPAT >6-OH-DPAT to no glucuronidation at all for 8-OH-DPAT. Thus, the position of the phenolic hydroxy group is an important factor for the UDPGT activity. Similar observations were reported on methyl substituted phenols[2].

The intrinsic clearance of PHNO in rat microsomes at 24 $\mu l.min^{-1}.mg^{-1}$ is comparable to that of 5-OH-DPAT and 6-OH-DPAT. However, when aromatic substituents are being introduced at the propyl side chain in both the DPAT structure and the PHNO structure, a considerable increase is seen in the intrinsic clearance for the rat, with N-0434 and N-0437 now showing comparable clearances to that of 7-OH-DPAT. It should be noted that the relatively large standard errors for the K_m's of PHNO and N-0434, using rat and human liver microsomes, respectively, are mainly due to noise factors that occur under the measuring conditions for these substances.

When considering the results with human microsomes, it is clear that the latter appear to be less active than the rat microsomes. This may have been due to the fact that the human material available was from, a 62-years old person but differences in isoenzymes between human and rat microsomes may also exist[20]. Nevertheless the human microsomes appear to show the same trend as in the rat, namely that aromatic substitutions at the propyl side chain dramatically increase glucuronidation rate. Introduction of a chlorine atom at the 8-position in N-0437, making the 5-hydroxy group more acidic resulted in an even higher clearance, which is in accordance with previous observations[2]. It can be concluded, therefore, that although the affinity towards the dopamine receptor of the hydroxy-2-(N,N-dipropylamino)tetralins can be enhanced by the substitution of a propyl side chain in a thienylethyl- or phenylethyl side chain, the latter also increases the metabolic inactivation by glucuronidation. This may negatively affect the ability of these drugs to reach the target tissues in the brain.

Table II. Kinetic parameters of human and rat microsomal UDP-glucuronosyltransferase activity towards the hydroxy-2-aminotetralins and naphthoxazines. Values shown are the means of three experiments ± S.E.

	human liver microsomes			rat liver microsomes		
	V_m nmol.min^{-1}.mg^{-1} protein	K_m μM	V_m/K_m μl.min^{-1}.mg^{-1} protein	V_m nmol.min^{-1}.mg^{-1} protein	K_m μM	V_m/K_m μl.min^{-1}.mg^{-1} protein
N-0437	3.6 ± 1.0	292 ± 79	12.4 ± 0.4	2.6 ± 0.2	8.9 ± 0.72	91 ± 18
N-0434	1.4 ± 0.7	271 ± 145	5.0 ± 0.5	2.2 ± 0.1	9.0 ± 1.1	244 ± 26
PHNO		no glucuronidation		6.4 ± 4.0	261 ± 165	24.4 ± 1.7
T-PHNO	3.0 ± 0.3	249 ± 15	11.8 ± 0.5	6.2 ± 0.2	65.4 ± 1.4	95.3 ± 7.6
5-OH-DPAT		no glucuronidation		3.1 ± 0.6	87.5 ± 0.6	35.6 ± 1.7
6-OH-DPAT		no glucuronidation		5.1 ± 2.7	470 ± 3	10.9 ± 1.1
7-OH-DPAT		no glucuronidation		17.8 ± 1.0	57.4 ± 1.6	311 ± 22
8-OH-DPAT		no glucuronidation			no glucuronidation	
Cl-N-0437	5.3 ± 1.0	191 ± 38	27.9 ± 1.5		not determined	

REFERENCES

1. Boutin, J.A., Thomassin, J., Siest. G., and Cartier, A., *Biochem. Pharmacol.* **34**: 2235-2249 (1985).
2. Mulder, G.J., and Van Doorn, A.B.D., *Biochem. J.* **151**: 131-140 (1985).
3. Okulicz-Kozaryn, J., Schaefer, M., Batt, A.M., Siest, G., and Loppinet, V., *Biochem. Pharmacol.* **30**: 1457-1461 (1981).
4. Thomassin, J., Cartier, A., Boutin, J.A., Magdalou, J., Siest, G., Costa, M.T., and Loppinet, V. *Eur. J. Med. Chem.* **22**: 393-402 (1887).
5. Seiler, M.P., and Markstein, R., *Mol. Pharmacol.* **22**: 281-289 (1982).
6. Coleman, R.J., Lange, K.W., Quinn, N.P., Loper, A.E., Bondi, J.V., Hichens, M., Stahl, S.M., and Marsden, C.D., *Movement Disorders* **4**: 129-138 (1989).
7. Löschmann, P.A., Chong, P.N., Nomoto, M., Tepper, P.G., Horn, A.S., Jenner, P., and Marsden, C.D., *Eur. J. Pharmacol.* **166**: 373-380 (1989).
8. Koller, W., Herbster, G., and Gordon, J., *Movement Disorders* **2**: 193-199 (1987).
9. Gerding, T.K., Drenth, B.F.H., Roosenstein, H.J., De Zeeuw, R.A., Tepper, P.G., and Horn, A.S., *Xenobiotica* **20**: 515-524 (1990).
10. Gerding, T.K., Drenth, B.F.H., De Zeeuw, R.A., Tepper, P.G., and Horn, A.S., *Xenobiotica* **20**: 525-536 (1990).
11. Beaulieu, M., Itoh, Y., Tepper, P., Horn, A.S., and Kebabian, J.W., *Eur. J. Pharmacol.***105**: 15-21 (1984).
12. Dijkstra, D., Hazelhoff, B., Mulder, T.B.A., De Vries, J.B., Wijnberg, H., and Horn, A.S., *Eur. J. Med. Chem.* **20**: 247-250 (1985).
13. Horn, A.S., Tepper, P., Van der Weide, J., Watanabe, M., Grigoriades, D. and Seeman, P.,*Pharm. Weekb. [Sci] Ed.* **7**: 208-211 (1985).
14. McDermed, J.D., McKenzie, G.M., and Philips, A.P., *J. Med. Chem.* **18**: 362-367 (1975).
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randell, R.J., *J. Biol. Chem.* **193**: 265-275 (1951).
16. Väisänen, M.V.T., Mackenzie, P.I. and Hänninen, O.O.P., *Eur. J. Biochem.* **130**: 141-145 (1983).
17. Dumont, E., Von Bahr, C., Perry, T.L., and Bertilsson, L., *Pharmacol. Toxicol.* **61**: 335-341 (1987).
18. Coughtrie, M.W., Ask, B., Rane, A., Burchell, B., and Hume, R., *Biochem. Pharmacol.* **38**: 3273-3280 (1989).
19. Gerding, T.K., Drenth, B.F.H., Van de Grampel, V.J.M., Niemeyer, N.R., De Zeeuw, R.A., Tepper, P.G., and Horn, A.S., *J. Chromatogr.* **487**: 125-134 (1989).
20. Tephly, T.R., Townsend, M., and Green, M.D., *Drug Metabolism Reviews* **20**: 689-695 (1989).